

Hoeller U, Baur M, Roos FF, Brennan L, Daniel H, Fallaize R, Forster H, Gibney ER, Gibney M, Godlewska M, Hartwig K, Kolossa S, Lambrinou CP, Livingstone KM, Lovegrove JA, Macready AL, Manios Y, Marsaux CFM, Martinez JA, Celis-Morales C, Moschonis G, Navas-Carretero S, O'Donovan CB, San-Cristobal R, Saris WHM, Surwillo A, Traczyk I, Tsirigoti L, Walsh MC, Woolhead C, Mathers JC, Weber P, Food4Me Project.

[Application of dried blood spots to determine vitamin D status in a large nutritional study with unsupervised sampling: the Food4Me project.](#)

*British Journal of Nutrition* 2016, 115(2), 202-211

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This is the authors accepted manuscript of an article that has been published in its final definitive form by Cambridge University Press, 2016

**DOI link to article:**

<http://dx.doi.org/10.1017/S0007114515004298>

**Date deposited:**

13/04/2016

**Embargo release date:**

09 May 2016



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# Application of Dried Blood Spots to determine vitamin D status in a large nutritional study with unsupervised sampling: the Food4Me project

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## Abstract

An efficient and robust method to measure vitamin D (25-OH-D3 and 25-OH-D2) in dried blood spots (DBS) has been developed and applied in the pan-European multi-centre, internet-based personalized nutrition intervention study Food4Me. The method includes calibration with blood containing endogenous 25-OH-D3, spotted as DBS and corrected for haematocrit content. The methodology was validated following international standards. The performance characteristics did not reach those of the current gold standard LC-MS/MS in plasma for all parameters, but were found to be very suitable for status level determination under field conditions. DBS sample quality was very high, and 3778 measurements of 25-OH-D3 were obtained from 1465 participants. The study centre and the season within study centre were very good predictors of 25-OH-D3 levels ( $p < 0.001$  for each case). Seasonal effects were modelled by fitting a sine function with a minimum 25-OH-D3 level at January 20, and a maximum at July 21. The seasonal amplitude varied from centre to centre. The largest difference between winter and summer levels was found in Germany and the smallest in Poland. The model was cross-validated to determine the consistency of the predictions and the performance of the DBS method. The Pearson correlation between the measured values and the predicted values was  $r = 0.65$  and the standard deviation of their differences was 21.2 nmol/l. This includes the analytical variation, and the biological variation within subjects. Overall, DBS obtained by unsupervised sampling of the participants at home was a viable methodology for obtaining vitamin D status information in a large nutritional study.

60

## 61 Introduction

62 Dried blood spotting is a minimally invasive technique to obtain blood samples on cards of  
63 filter paper for biochemical or genetic analysis. A small prick in a fingertip is enough to  
64 produce a drop of blood suitable for sampling. This approach has the advantages of being  
65 considerably less invasive than venous blood sampling, does not require health  
66 professionals and can be performed by most people on themselves after relatively little  
67 training. In addition, if the analyte is stable in dried whole blood, samples can be  
68 transported at room temperature by regular mail. Infrastructure for taking venous blood  
69 samples, separation of plasma, and storage and shipment of frozen samples is no longer  
70 required. In practice, blood sampling can be achieved anywhere and at any time. This offers  
71 significant benefits, especially for nutrient status determination in populations at remote  
72 locations or at home<sup>(1,2)</sup>.

73 These advantages made the methodology ideal for use in an internet-based intervention,  
74 such as the Food4Me study, where all data were self-reported and biological samples were  
75 collected remotely by the participants<sup>(3)</sup>. To date, the Food4Me study is the largest multi-  
76 centre, internet-based personalized nutrition intervention aiming to compare the  
77 effectiveness of three levels of personalized nutrition (based on dietary, phenotypic and  
78 genotypic data) on behavioural changes (diet and physical activity) and health outcomes  
79 (blood metabolites and obesity-related anthropometrics)<sup>(3)</sup>. The DBS technique was first  
80 introduced for diagnosing phenylketonuria in new-borns by measuring whole blood  
81 phenylalanine concentration<sup>(4)</sup>, and has since been applied to multiple metabolites<sup>(1,5)</sup>. A  
82 critical success factor for assessment of many vitamins and other micronutrients in DBS is  
83 their stability on the sampling cards. The compounds need to be stable in the presence of  
84 oxygen at ambient temperature for at least the initial drying time and shipment time to the  
85 laboratory. In the case of the Food4Me study, this period was about one week. Stability can  
86 be increased by adding reagents such as antioxidants to the sampling cards. However, this  
87 approach has limitations due to safety concerns as the impregnated card comes in close  
88 contact with the pricked fingertip. To date, DBS assays reported for micronutrients include  
89 the vitamins A<sup>(6,7)</sup>, B<sub>12</sub><sup>(8)</sup>, D<sup>(9-13)</sup>, K<sup>(14)</sup> and folate<sup>(15)</sup>. However, only the assays for vitamin A

and D appear to be used frequently. While vitamin A was reported to be somewhat unstable with a loss of >10% over the first week<sup>(16)</sup>, no stability issues have been reported for vitamin D (25-hydroxy vitamin D3). One explanation for the particular stability of this vitamin may be the presence of a specific vitamin D binding protein in blood that, together with serum albumin, bind >99 % of the circulating 25-hydroxy vitamin D<sup>(17,18)</sup>, thereby stabilizing the vitamin on the DBS cards. Vitamin D assays based on DBS have been reported by three independent research groups viz., Eyles et al.<sup>(9)</sup> and Newman et al.<sup>(11)</sup> in 2009, and by Higashi et al. in 2011<sup>(12)</sup>. While all methods focus on 25-hydroxy vitamin D3 (25-OH-D3), some procedures also include related metabolites, including 25-hydroxy vitamin D2 (25-OH-D2) and 3-epi-25-hydroxy vitamin D3. The main differences between the assays are in the detection and calibration methods used and the extent of validation data that have been published. To date, all methods have been based on reversed-phase LC-MS/MS with ESI<sup>+</sup> detection. Eyles and Higashi derivatised the analytes with a nitrogen containing Diels-Alder reagent (4-phenyl-1,2,4-triazoline-3,5-dione, PTAD) which increases the sensitivity during MS-detection significantly, but has two drawbacks: it introduces an additional time consuming step during sample preparation, and the derivatisation leads to formation of two stereoisomers that complicate chromatography. The method by Newman et al.<sup>(11)</sup> does not use analyte derivatisation, but starts with four large punches of filter paper that limit automated handling in small vials during sample preparation. A later publication from the same group indicates that the method has been modified and now also includes derivatisation of the analytes<sup>(13)</sup>. However, this approach requires by far the largest sample aliquots i.e. four 6 mm diameter punches.

In general, calibration of DBS analysis is difficult due to the absence of blank samples in the case of endogenous analytes, and the fact that current reference analytics is performed from plasma or serum samples, not (dried) blood. All existing methods for vitamin D analysis share a quite tedious calibration approach using spiked samples of blood, vitamin D depleted blood or plasma for calibration.

Our aim was to develop a novel, quicker assay for 25-OH-D3 and 25-OH-D2 from DBS without chemical derivatisation to cope with the expected large sample numbers from the Food4Me study and which might have utility for future large studies and surveys. We focused particularly on improving the calibration methodology to allow direct comparison of

the results with published vitamin D status data, which are derived typically from measurements on plasma. Aspects of method development are reported, together with method validation and performance data. The Food4Me study did not include venous blood sampling, which precluded direct comparison of the results obtained from unsupervised collection of DBS by the participants at home with a reference method. Quality measures including spot quality and consistency of status levels of each participant measured at each time point have been assessed. The vitamin D status results were correlated with variables such as seasonality and research centre, and compared with literature data.

## Materials and methods

### *Study design and participants<sup>(3)</sup>*

The Food4Me intervention study was designed as a pan-European randomized controlled trial (RCT) to determine whether providing personalized dietary advice leads to greater improvements in eating patterns and health outcomes compared with a conventional population-based general guidelines approach. Seven research centres in seven European countries participated with more than 220 participants from each centre. DBS samples were taken and analysed at three time-points: at baseline, and after 3 and 6 months intervention, respectively. Details of the study design and baseline characteristics of the participants have been published elsewhere<sup>(3)</sup>. The seven participating study centres were located in Munich (Germany), Athens (Greece), Dublin (Ireland), Maastricht (The Netherlands), Warsaw (Poland), Navarra (Spain) and Reading (U.K.), and recruitment was done country-wide. A total of 5562 participants (65 % females) were screened online over a 12 month period between August 2012 and August 2013 and consented to participate. Of these, 1607 (28.9%) were recruited into the RCT. Participants aged 18 to 79 years (mean 39.8, SD 13.1) were included in the study of whom 60.9% (n= 980) were women and 96.7% were from white-European background. The mean BMI for all participants was 25.5 (SD 5.2) kg/m<sup>2</sup> and 44.8% (n= 721) of participants were overweight or obese (BMI  $\geq$  25.0 kg/m<sup>2</sup>). This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the ethics committees of the participating centres. Written or online informed consent was obtained from all participants.

Further details on study design and participant characteristics can be found in Celis-Morales et al.<sup>(3,19)</sup>

#### *Samples and sampling protocol*

Finger-prick blood samples were collected by participants themselves using a collection pack with two cards, one provided by Vitas Ltd, Oslo, Norway and one by DSM, Kaiseraugst, Switzerland. Previous to spotting blood, cards for vitamin D analysis (Whatman Protein Saver 903 Card, GE Healthcare, Westborough, MA) were pre-treated with 1 % of 2,6-di-*tert*-butyl-4-methylphenol (BHT) dissolved in methanol (MeOH). 30 µl of 1 % BHT in MeOH were pipetted to each circle on the card and allowed to dry for at least 30 min at room temperature (RT). These pre-treated cards were packed in an airtight aluminium bag (Whatman Foil Bags, item no. 10534321, Whatman Inc., Sanford, ME) with a drying agent (Sorb-it, item no. 10548234, Süd-Chemie, Germany) and stored at room temperature until use. To help with blood collection, participants had access to an online video demonstration with written instructions and frequently asked questions in the language of recruitment. For the finger pricks, 2.0 mm contact-activated lancets (BD Microtainer, Becton, Dickinson and Company, Franklin Lakes, NJ) were used. Each participant was asked to fill the two dry blood spot cards each with five spots at each collection time-point. This is the approx. equivalent of five drops of blood or 250 µl of blood per card. When the 10 blood spots were filled, participants were instructed to let the cards dry at RT for at least 2 hours, but not longer than 4 hours, before samples were put back into the aluminium bags and returned by mail to the corresponding recruiting centre. The centres verified the content of the bags, and shipped one bag containing the DBS card to DSM (DSM Nutritional Products Ltd. Switzerland) for measurements of vitamin D (25-OH D2 and 25-OH D3). While the shipments were done at ambient temperature, the closed bags were stored at the centres and at DSM at nominal -20°C.

Calibration was done with whole blood received from blood donors of the "Blutspendezentrum SRK beider Basel" [blood donation centre at Basel hospital], including haematocrit values for each sample. The calibration samples (n = 15) had a mean haematocrit content of 44.2 % (range 38.7 - 49.3 %). Donors were 12 females and 3 males



with an average age of 49 years. The calibration samples were prepared as following: blood aliquots of 50 µl were pipetted onto a card and allowed to dry for 2 - 4 hours at RT, avoiding exposure to direct sunlight. Cards were then transferred into aluminium foil bags with desiccant inside and stored in a freezer at nominal -20°C. Calibration samples were used for 3 - 6 months. As quality control, DBS samples with independently determined 25-OH-D3 content were analysed within each analytical run.

### *Reagents and instruments*

25-Hydroxycholecalciferol monohydrate (25-OH-D3 monohydrate) was from Dr. Ehrenstorfer, Augsburg, Germany. 25-Hydroxyergocalciferol (25-OH-D2) and sodium dodecyl sulphate ( $\geq 99\%$ , SDS) were supplied by Sigma-Aldrich, St. Louis, USA, 26,26,26,27,27,27-hexadeutero-25-hydroxycholecalciferol (25-OH-D3-d6) was supplied by Medical Isotopes, Pelham, USA, 2,6-di-*tert*-butyl-4-methylphenol ( $\geq 99\%$ , BHT), acetonitrile (gradient grade), formic acid (Suprapur), toluene and methanol (p.a., MeOH) were from Merck, Darmstadt, Germany, and MS-grade water was prepared on a Milli-Q instrument. Eppendorf tubes were centrifuged on a 5417R model centrifuge (Vaudaux-Eppendorf, Schönenbuch, Switzerland), and evaporation was done on a Cyclone (Prolab, Reinach, Switzerland). Analyte separation was performed on an Agilent 1260 HPLC with auto sampler, two binary pumps and column oven coupled to an AB Sciex Qtrap 5500 MS/MS-System with APPI source.

### *Assay*

#### *Sample preparation:*

Prior to analysis, the samples were assessed to see if they met the quality criteria (Figure 1): spot size (circle filled), thoroughly soaked (observed from the back), and one application of blood (not composed of many small spots). Two punches (inner diameter of 3.175 mm) were taken out of the card and placed into a 2 ml Eppendorf tube. After adding 100 µl of 0.1 % SDS-solution and 20 µl of internal standard (ISTD, 25,25,25,26,26,26-hexa-deutero-25-OH-D3, 25 ng/ml in methanol) the tubes were shaken at 40°C for 30 min. Then, 400 µl of

209 acetonitrile was added and the shaking continued at RT for 5 min (260/min, IKA shaker).  
210 Afterwards, the tubes were centrifuged at 20000 g for 5 min and the supernatant  
211 transferred into a new 2 ml Eppendorf-tube. The solvent was evaporated to dryness under  
212 vacuum (Cyclone) and the residue was reconstituted with 50 µl injection solvent  
213 (methanol/water, 70:30) and transferred into a micro vial for analysis.

#### 214 Chromatography and detection:

215 Chromatography was performed on an Ascentis Express C18 column, 7.5 x 2.1 mm, 2.7 µm  
216 with a guard column using the following gradient: 0 min, flow 600 µl/min, 15 % A; 1.8 min,  
217 600 µl/min, 0 % A; 1.9 min, 1000 µl/min, 0 % A; 3.7 min, 1000 µl/min, 0 % A; 3.8 min, 1000  
218 µl/min, 15 % A; 5.8 min, 1000 µl/min, 15 % A; 5.9 min, 600 µl/min, 15 % A; 6.0 min, 600  
219 µl/min, 15 % A. Mobile phase A consisted of water containing 0.05 % formic acid; mobile  
220 phase B consisted of methanol/acetonitrile (80/20, v/v) containing 0.05 % formic acid.

221 Samples were kept at 10°C, column temperature was 30°C and injection volume was 10 µl.  
222 Dopant for APPI detection was toluene added post-column at 100 µl/min. Typical retention  
223 times were 1.55 min (25-OH-D3 and ISTD) and 1.71 min (25-OH-D2).

224 Detection was done on an AB Sciex 5500 Qtrap instrument with APPI positive mode and  
225 MRM scan type at unit resolution.

226 Parameter table: CUR 20; CAD Medium (9); IS 775; TEM 320; GS1 75; GS2 50; EP 10.

227 25-OH-D3, 383.3 Q1 Mass (Da), 211.3 Q3 Mass (Da), 50 Dwell (msec), 66 DP, 35 CE, 8 CXP;  
228 25-OH-D3 qualifier, 383.3 Q1 Mass (Da), 257.2 Q3 Mass (Da), 50 Dwell (msec), 91 DP, 21 CE,  
229 12 CXP; ISTD 25-OH-D3-d6, 389.3 Q1 Mass (Da), 211.3 Q3 Mass (Da), 50 Dwell (msec), 51 DP,  
230 41 CE, 8 CXP; 25-OH-D2, 395.2 Q1 Mass (Da), 269.2 Q3 Mass (Da), 50 Dwell (msec), 46 DP,  
231 29 CE, 12 CXP; 25-OH-D2 qualifier, 395.2 Q1 Mass (Da), 209.2 Q3 Mass (Da), 50 Dwell (msec),  
232 56 DP, 39 CE, 10 CXP.

#### 233 Determination of nominal 25-OH-D3 content of calibration samples:

234 An aliquot of each whole blood sample spotted for calibration was used for preparation of  
235 plasma. The 25-OH-D3 content of this plasma was measured with an established reference  
236 method<sup>(20)</sup>. The haematocrit content of the whole blood used for calibration was measured

at the blood donation centre. As the haematocrit contents of the study samples were unknown, an estimated mean value of 40 % was used for calculation. This value was based on the rounded mean haematocrit content of the samples used for method development and validation. The following equation was used to normalize the calibration samples accordingly.

$$c(25 - OH - D3)_{normalised} = c(25 - OH - D3)_{measured} \times \frac{(100 - haematocrit)}{60}$$

$c(25\text{-OH-D3})_{normalised}$ : concentration of 25-OH-D3 (ng/ml plasma) in a calibration sample, normalized to 40 % haematocrit content

$c(25\text{-OH-D3})_{measured}$ : concentration of 25-OH-D3 (ng/ml plasma) measured with reference method in plasma samples obtained from whole blood used for calibration

*haematocrit*: haematocrit value (%) for whole blood sample obtained from Blutspendezentrum Basel

For study samples, the resulting 25-OH-D3 concentration was then corrected for sex-specific mean haematocrit values of 41.5 % for female and 46.5 % for male participants by applying correction factors of 1.026 for female and 1.121 for male. This was based on information on the sex-specific reference ranges and means obtained from the seven clinical centres (personal information).

#### *Performance criteria*

The method was validated based on the procedures described in the 'Guideline on bioanalytical method validation' of the European Medicines Agency<sup>(21)</sup> taking into account specific requirements and recommendations for DBS analysis<sup>(22)</sup>. Selectivity was tested by comparison of chromatograms from five samples of different donors with and without spike of either 25-OH-D3-d6 or 25-OH-D3 at 20 ng/ml blood. As the samples contained endogenous 25-OH-D3, area ratios of qualifier ion m/z [383 / 257] to quantifier ion m/z [383 / 211] were calculated and used for assessment of the selectivity of the detection of 25-OH-D3. Carry-over was assessed by measuring blank samples after analysis of high content samples. No significant carry over was observed. Linearity was determined by analysis of calibration samples according to the method on three different days. Linear regression of

the analyte peak area ratio (analyte peak area versus internal standard peak area) against the nominal concentration was calculated. Differences between nominal concentrations and calculated concentrations were determined and expressed in %. Deviations of the measured values from nominal values should be  $\leq 20\%$  and at least 2/3 of the calibration levels at each day should meet these criteria. As calibration was performed with endogenous samples, only a limited concentration range could be tested: 32.5 – 120 nmol/l calculated as plasma concentrations. The lower limit of quantification was set to 25 nmol/l as this represents the accepted cut off for vitamin D deficiency<sup>(23)</sup>. Accurate determination of lower concentrations was not within the scope of the study. For assessing accuracy, samples of five different subjects were analysed. Prior to spotting the whole blood samples, plasma was separated from a whole blood aliquot of each sample and analysed with a reference method<sup>(24)</sup>. Nominal DBS concentrations were calculated by correcting the plasma concentrations with the corresponding haematocrit value (nominal concentration = plasma concentration  $\times$  (100 – haematocrit value) / 100). DBS samples from each subject were analysed in fivefold and compared with the nominal DBS concentration. Mean accuracy was calculated and reported as percentage of nominal DBS concentration value. As additional measure for accuracy and of the influence of varying haematocrit values in the calibration samples, we re-analysed calibration samples of which the 25-OH-D3 concentration values were assigned previously within a new set of calibration samples with independently assigned 25-OH-D3 concentration values. The mean haematocrit value of these samples was 42.0 % (range 39.6 - 43.8 %). To determine intra-day precision, the results from samples prepared for accuracy were used. To estimate inter-day precision, triplicates of each sample were analysed on 3 different days. Precision was reported as coefficient of variation (CV) of the measured concentrations. For determination of stability in the auto sampler at 10°C, extracts of incurred and spiked DSB samples were re-analysed after 60 h. Long-term stability was tested by storing DBS samples for up to 6 months at nominal -20°C and comparing the content analysed after the storage with starting values. There was good stability under both conditions.

#### *Statistical analysis*

Longitudinal linear mixed models were used to model 25-OH-D3 levels. Limit of detection values (LOD) in the 25-OH-D3 measurements were replaced by 12.5 nmol/l (four measurements, 0.1 %) and lower limit of quantitation values (LOQ) were replaced by 16.25 nmol/l 25-OH-D3 (134 measurements, 3.5 %). Sensitivity analyses were carried out assuming 0 nmol/l for LOD and LOQ values, and 25 nmol/l for LOD and LOQ values, respectively. For the centre with by far the most LOQ values (Dublin), the model fits differed by -4.0 nmol/l in winter, assuming 0 nmol/l for LOD and LOQ, and +0.2 nmol/l in summer. Assuming 25 nmol/l for LOQ and LOD values the difference was +2.2 nmol/l in the winter and -0.1 nmol/l in the summer. The absolute deviations for all other centres were  $\leq 2.4$  nmol/l in the winter and  $\leq 0.2$  nmol/l in summer.

To model the seasonal variation at the study sites, the study centre and the interaction of study centre with the functions  $\sin(\text{SampleYear} \times 2 \times \pi)$  and  $\cos(\text{SampleYear} \times 2 \times \pi)$  were included as fixed effects, and the participants as a random effect. The 20<sup>th</sup> January was found to be the consensus date across all study centres when the 25-OH-D3 concentrations reached their nadir. To simplify the subsequent modelling and interpretation, a single normalized sine function was derived which oscillated between -1.0 when the 25-OH-D3 concentration was at its lowest on the 20<sup>th</sup> of January and +1.0 when the 25-OH-D3 concentration was at its highest on the 21<sup>st</sup> of July. It assumes synchronized timing of seasons across all study centres, but it differentiates mean levels and seasonal amplitudes by study centre. The function was coded as follows:

StandardizedSeasonalAmplitude=SSA= $\sin((\text{SampleYear}-20/365.25-0.25) \times 2 \times \pi)$ . On the 20<sup>th</sup> of each month, this function had the following values: -1.0 in January, -0.9 in February, -0.5 in March, 0.0 in April, 0.5 in May, 0.9 in June, 1.0 in July, 0.9 in August, 0.5 in September, 0.0 in October, -0.5 in November, and -0.9 in December. For a sample taken on the 30<sup>th</sup> of June, the SampleYear would equal 2013.5, and the resulting SSA would be 0.94. For each measurement, the corresponding SSA was thus calculated. The final model was then fitted using centre and the centre-SSA interaction as fixed effects, and participant ID as random effect (presented in Table 2 and Figure 2). Due to the absence of a gold standard method, the biological and the analytical variability could not be separated in the present data set. The overall variation should thus be considered as a highly conservative estimate of the variation of the analytical method. R statistics software version 3.02 was used for all

statistical analyses,<sup>(25)</sup> and within the R statistics software, the function lme() in the package nlme<sup>(26)</sup> was used for the mixed model regressions.

## Results

The assay described here was developed for analysis of the large sample numbers from the Food4Me study. Calibration was performed with blood samples containing endogenous analyte content spotted on DBS cards. The nominal 25-OH-D3 concentration of these calibration samples was determined from plasma obtained from the same samples with an established reference method, corrected by the measured haematocrit value. Stability trials showed that these calibration samples could be used for at least 6 months if stored at nominal -20°C. The current method is equally applicable for determination of 25-OH-D2 but because substantial concentrations of 25-OH-D2 were not expected in the current study, method validation focused on 25-OH-D3. An overview of parameters validated and results are given in Table 1. The re-analysis of calibration samples within an independent calibration showed a good correlation between the originally assigned 25-OH-D3 concentrations and the concentrations determined from the independent calibration for these samples (n = 5, R = 0.97). Overall, the DBS method showed results with slightly larger variations compared with current reference methodologies but within or close to the acceptance ranges of the guidelines. This led to specific characteristics of e.g. a rather small linear range of 32.5 - 120 nmol/l with deviation of up to 18 % between measured and nominal values (guideline limit 15%), and precision up to 13-14 % CV for inter- and intra-day. Overall, it was concluded that the method was suitable for determination of status levels for vitamin D and thus for application in the Food4Me study.

In total 3778 DBS samples were analysed: 453 from Germany, 560 from Greece, 554 from Ireland, 634 from The Netherlands, 530 from Poland, 555 from Spain and 492 from the U.K. From 1003 participants, DBS at three time points could be measured, from 307 participants two time points could be measured, while for 155 participants only one time point could be measured. In addition, no samples were received for analysis from 259 participants (19.7 %

of total cohort who completed the study), and whilst a further 67 samples gave a valid result for 25-OH-D3 concentration they lacked a sampling date and so could not be included in the analysis of effects of seasons. Only 80 samples either did not meet the quality criteria or had insufficient blood spots for analysis of all required parameters, and only five analyses failed due to technical errors. Overall the quality of the samples was very good, showing that the methodology is suitable for unsupervised sampling at home following provision of detailed instructions to the participants including e.g. a video demonstration of the sampling process.

The data for 25-OH-D3 concentration are presented in Figure 2. None of the samples contained 25-OH-D2 above the LOQ of 25 nmol/l. The seasonality of the 25-OH-D3 status for the participants of each study centre is clearly visible ( $p < 0.001$ ) with significantly higher concentrations in summer than in winter (67.1-92.1 vs. 38.0-63.0 nmol/l). Details of the observed correlations are given in Table 2. Due to the different starting dates, and the different duration of the study due to recruiting progress in each centre, the coverage of the annual time period varied between centres, but it usually included the seasonal minimum and maximum. DBS collection over more than 12 months occurred in only two centres, Dublin in Ireland and Reading in the U.K. The values for the seasonal minima and maxima of 25-OH-D3 levels varied by country (Figure 2). The largest seasonal changes were observed in the participants from Germany 92.1 nmol/l in summer vs. 41.9 nmol/l in winter, whereas the smallest changes were recorded in Poland (67.1 nmol/l vs. 50.4 nmol/l, respectively). Overall, the highest values were found in late summer (July 21), and the lowest values in late winter (January 20). This is in good agreement with data from the literature, although the number of reports from comparable cohorts is limited<sup>(27)</sup>.

To assess the performance of the analytical method and the statistical model, a leave-one-out cross-validation was performed. Only participants for whom all three planned measurements were available were included in the validation. For each measured value a prediction was performed by taking into account the site and the season as fixed factors, and the subject ID as a random factor. The two other measurements for the same participant were included each time, and the measurement to be predicted was excluded each time. In the absence of a gold standard, these predicted values were considered as the most plausible reference values against which the actual measurement values could be compared. The resulting Pearson correlation between measured and modelled values was

r=0.65, and the differences between the modelled and measured values had a standard deviation of 21.2 nmol/l. This variation is attributable both to biological and analytical variation.

## Discussion

With 3778 valid measurements obtained from 1465 participants, to the best of our knowledge the Food4Me study is the largest study using DBS samples where the participants did the sampling themselves at home. Sample preparation was designed to be as fast and as easy as possible, with the potential to perform all necessary steps in a multiwell-plate. A crucial step for the whole methodology was the calibration of the assay. While analysis of 25-OH-D3 from DBS has been applied before, we introduced improvements including calibration using DBS with endogenous analyte content and corrected by the haematocrit content of the calibration samples. As 25-OH-D3 is an endogenous compound, no blank samples of blood were available. In addition, spiking of blood with 25-OH-D3 was considered to be questionable, as the endogenous analyte is almost completely protein bound, and conditions to simulate this with spiking were difficult to achieve. Using blood with endogenous 25-OH-D3 content simplifies the calibration procedure and makes the methodology more robust as unknowns and calibration samples consist of the same matrix. As reference values for many micronutrients, including vitamin D, are typically reported as plasma levels, a conversion from whole blood is needed. This conversion is based on the haematocrit content that varies from person to person<sup>(28)</sup>. As was the case in the Food4Me study, this value is often not available for study samples. Different approaches to circumvent this issue have been proposed<sup>(29)</sup> which typically require an additional measurement of a second blood component such as potassium<sup>(30)</sup>, for normalization, an approach which introduces additional analytical variance. For the current study, a haematocrit value of 41.5 % was assigned to samples from female subjects and a value of 46.5 % to samples from male subjects. As the study design did not include the concurrent sampling of venous blood and the analysis for 25-OH-D3 in the resulting plasma as a reference, the influence of haematocrit on the accuracy of the results could not be verified independently. However, we estimated that our assumption introduced a small



amount of additional variation, perhaps <10 %. I.e., when considering an exemplified normal range of 36 - 47 % haematocrit for women, a plasma concentration of 50 nmol/l calculated for a mean of 41.5 % haematocrit could vary between 45.7 and 55.2 nmol/l. The influence of other factors including spot size and location of punches on vitamin D determination in DBS have been reported in the literature.<sup>(31)</sup> Whilst our new method did not reach the accuracy of the reference analytics of 25-OH-D3 from plasma by LC-MS/MS, the results are comparable with, or better than, those reported for other DBS-based methods. E.g., accuracy by recovery was reported in the range of 95.2 - 102.7 % (four concentration levels)<sup>(12)</sup> and 80 - 118 % (three levels)<sup>(9)</sup> and intra-assay precision in the range of 3.2 - 6.9 % RSD<sup>(12)</sup>, 8 - 13 % RSD<sup>(9)</sup> and 11 - 13 % RSD (three levels, inter-assay)<sup>(11)</sup>. Overall, the new method was found to be suitable for status level determination, as indicated by the performance criteria in comparison with international guidelines.

In the Food4Me study, sampling of the blood from the finger-tip by participants was done unsupervised. This posed questions of compliance with the sampling protocol, and with regard to the impact of deviations from the protocol on overall sample quality. We found that the large majority of blood spots were of very good quality when assessed visually compared with our quality control criteria. This also suggests that this finger prick approach to blood collection was well accepted by the participants given the total of acceptable measurements, and that provision of both written and video instruction ensures that blood samples can be obtained reliably from untrained participants in large cohort studies across multiple countries. Quality aspects not assessable by visual inspection include too long or too short drying times of the blood, and excessive sun or heat exposure of the sample before it reached the research centre. In addition, the identity of the blood donor could not be verified independently, although an embedded validation study of 10 % of participants, which included verification of identity based on genotype provided reassurance on this question<sup>(32)</sup>. Therefore, the consistency of data points for each individual participant was used as an indirect quality indicator. The resultant sequential assessment of vitamin D status from three DBS measurements over 6 months is unique. A rapid increase in vitamin D status is possible in case of extended sun exposure or the use of high dose supplementation, but a fast decline is very unlikely because 25-OH-D3 has a half-life of approx. 20 days following oral administration<sup>(33)</sup>. The demonstrated consistency of consecutive data from the

individual participants proves the robustness of the methodology, and the suitability of the DBS approach with unsupervised sampling for status determination of vitamin D3.

As expected, the results for all countries showed a strong seasonality with lowest mean vitamin D3 status levels observed towards the end of January and highest mean status levels towards the end of July. While representative data for vitamin D status is available in some European countries<sup>(27)</sup>, data on seasonality is more limited. For the U.K., lowest level of vitamin D3 concentrations were reported in February and highest levels in September during the 2002 - 2004 period<sup>(34)</sup>, with a difference of approx. 35 nmol/l. Furthermore, reports for Germany<sup>(35,36)</sup> clearly demonstrate a seasonality with the highest median value in June and the lowest median value in March with a difference of 23.4 nmol/l for 1998<sup>(36)</sup>. Although the participants of the current study may not be representative for each country, our results are comparable with the published data. An in-depth analysis of differences between countries will be done separately.

Overall, the current investigation provided valuable insight into effects of seasonality of vitamin D3 status in 1465 participants from seven European countries. The DBS-based methodology of unsupervised sampling of DBS by the participants at home was found to be suitable for status determination of 25-OH-D3 in the setting of the large, international nutritional study Food4Me. This encourages application in future studies, and has the potential for simultaneous determination of other micronutrients from the same DBS samples.

## Acknowledgements

This project was supported by the European Commission under the Food, Agriculture, Fisheries and Biotechnology Theme of the 7th Framework Programme for Research and Technological Development, grant number 265494.

The authors' contributions are as follows: U.H. drafted the manuscript and developed the analytical method together with M.B. who did all sample measurements; F.F.R. did the statistical analysis; P.W. contributed to the design of the analytical methodology and the data interpretation, J.C.M. was the study director of the proof of principle study of Food4Me;

H.D., M.G., J.A.L., Y.M., J.A.M., W.H.M.S., and I.T. contributed to the design of the proof of principle study and were principle investigator for their respective research centre; L.B., R.F., H.F., E.R.G., M.G., K.H., S.K., C.P.L., K.M.L., A.L.M., C.F M.M., C.C.-M., G.M., S.F.N.-C., C.B.O'D., R.S.-C., A.S., L.T., M.C.W., C.W. contributed to the study design and execution at the research centres. All authors contributed to, read and approved the final version of the manuscript.

UH, MB, FR and PW are employed by DSM Nutritional Products. The other authors have no potential financial or personal conflicts of interest to declare.

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Table 1: Overview of the performance characteristics of the analytical method. Details are given in the text.

Validation parameter	Procedure	Result (representative values)
Selectivity	Analysis of incurred and spiked samples	Interference with 25-OH-D3-d6 $\leq 0.4$ % Interference with 25-OH-D3 $\leq 14.2$ % (quantifier to qualifier ratio)
Carry-over	Injection of blank after high level spiked sample	Carry-over of 25-OH-D3 $\leq 1.6$ % Carry-over of ISTD $\leq 0.12$ %
Linearity	Analysis of incurred samples	32.5 – 120 nmol/l (as plasma concentration) Deviation measured from nominal value $\leq 18$ %; $R^2 \geq 0.95$
Accuracy	Incurred sample compared with reference value	80 – 95 %
Precision	Analysis of incurred sample	Intra-day: 5.8 - 13.0 % CV Inter-day: 8.4 - 14.0 % CV
Recovery	Analysis of incurred and spiked samples	74 - 101 %
Stability auto sampler (10°C)	Comparison with starting values	60 h at 10°C; difference: $\leq 6.1$ %
Stability long-term	Comparison with starting values	6 months at nominal -20°C; difference $\leq 14.1$ %

569 Table 2:<sup>1</sup> Association of 25-OH-D3 levels with the predictors "study centre" and "seasonal  
570 amplitude".

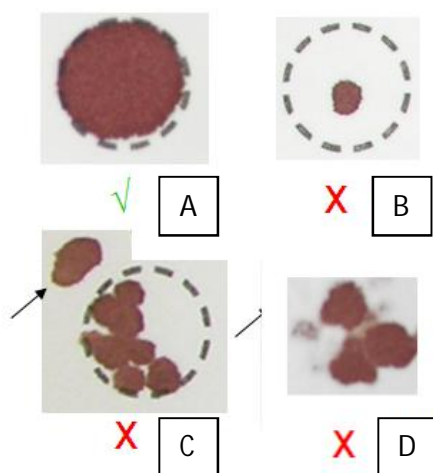
Location	Predictor	Coefficient [nmol/l]	Std.Error [nmol/l]	t-value	p	SeasonalMin [nmol/l]	SeasonalMax [nmol/l]
Munich, Germany	Study centre	67.0	1.5	43.6	<0.001	41.9	92.1
	Seasonal Amplitude	25.1	1.3	18.8	<0.001		
Athens, Greece	Study centre	60.7	1.5	39.9	<0.001	45.6	75.8
	Seasonal Amplitude	15.1	1.3	11.8	<0.001		
Dublin, Ireland	Study centre	57.1	1.4	40.3	<0.001	38.0	76.1
	Seasonal Amplitude	19.0	1.3	14.7	<0.001		
Maastricht , The Netherlands	Study centre	73.0	1.4	52.3	<0.001	63.0	83.1
	Seasonal Amplitude	10.0	1.1	8.9	<0.001		
Warsaw, Poland	Study centre	58.7	1.5	39.5	<0.001	50.4	67.1
	Seasonal Amplitude	8.4	1.2	6.7	<0.001		
Navarra, Spain	Study centre	65.2	1.5	42.2	<0.001	49.9	80.4
	Seasonal Amplitude	15.2	1.3	11.8	<0.001		
Reading, U.K.	Study centre	58.2	1.5	38.2	<0.001	42.1	74.2
	Seasonal Amplitude	16.1	1.4	11.3	<0.001		

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<sup>1</sup> The study centre and the season per study centre were very good predictors of the 25-OH-D3 levels. In Dublin for example, the model estimated that the 25-OH-D3 levels oscillate by amplitude of  $\pm 19.0$  nmol/l around a mean of 57.1 nmol/l over the seasons. This translates to a minimum of 38.0 nmol/l on the 20<sup>th</sup> of January, 57.1 nmol/l on the 20<sup>th</sup> of April, and a maximum of 76.1 nmol/l on the 21<sup>st</sup> of July.

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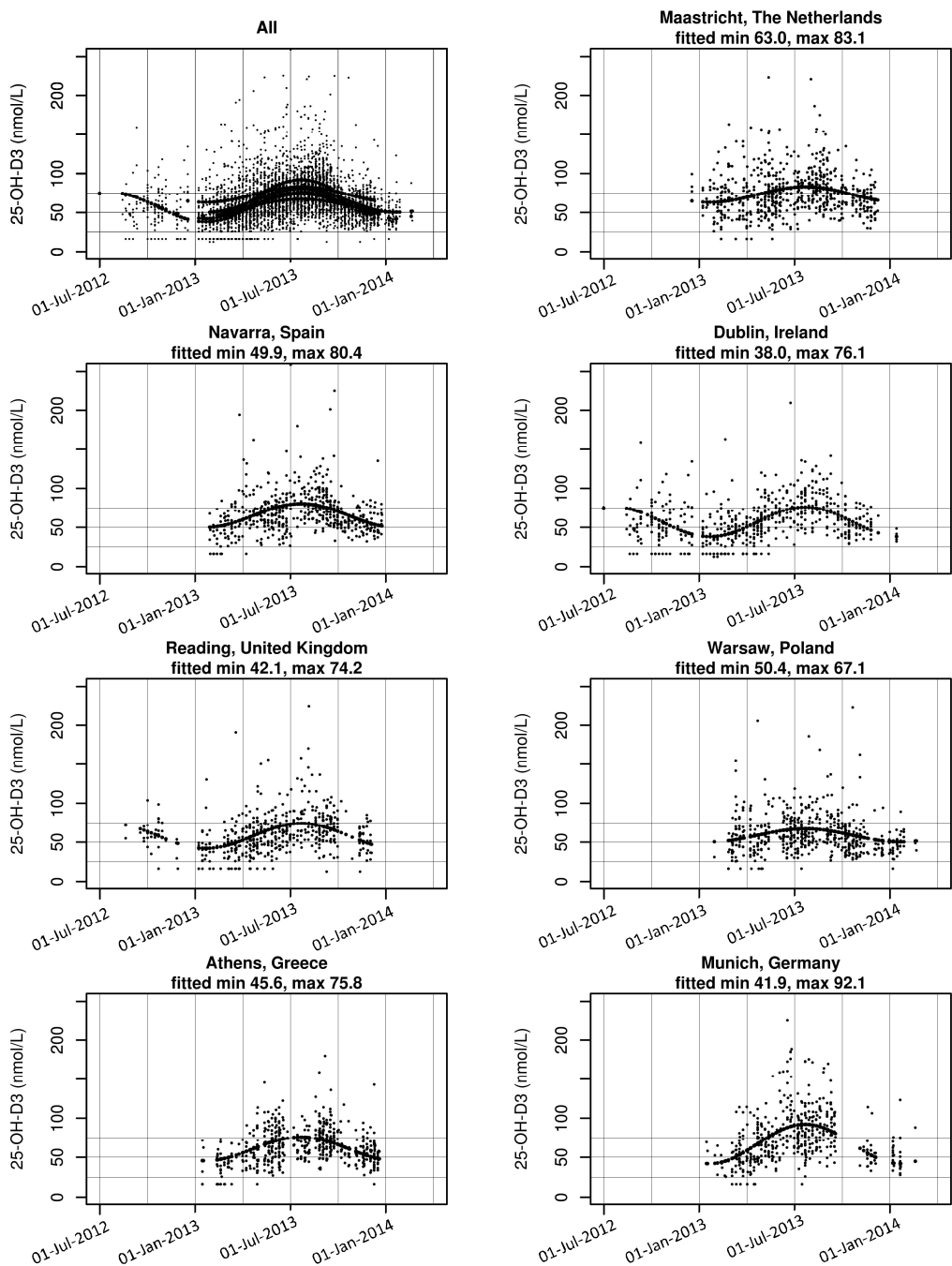


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575 Figure 1: Quality control criteria for dried blood spots from the Food4Me study. A: spot  
576 suitable for analysis. B - D: spots not suitable for analysis due to B: small spot size not filling  
577 the circle, C: multiple application of too small spots, including spots outside the circle, D:  
578 multiple application of small spots and no thorough soaking of the paper (view from the  
579 back).

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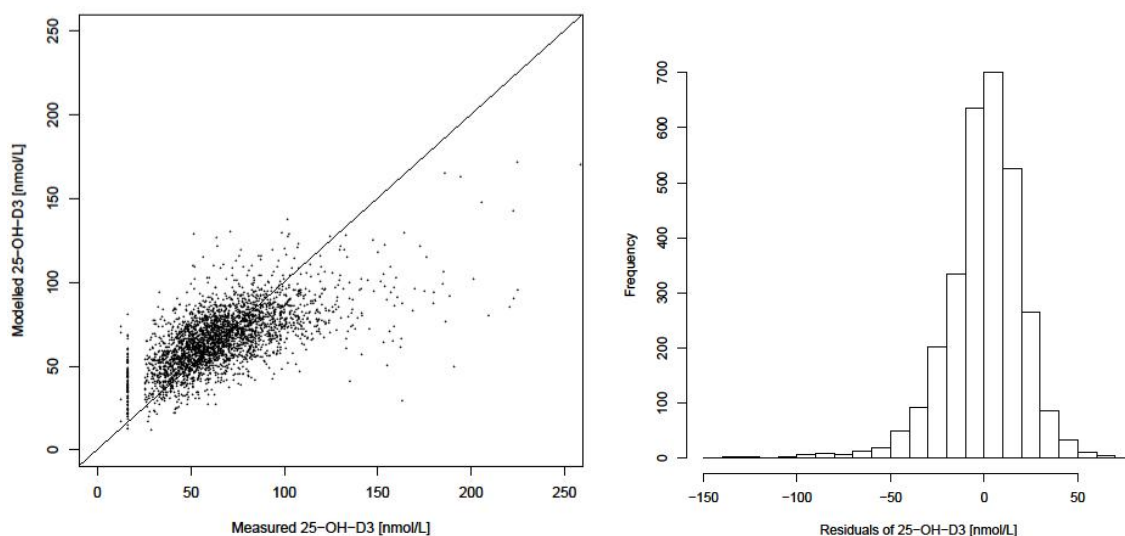
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Figure 2: Individual measurements for 25-OH-D3 by research centre and sampling date, and seasonal regression by centre. The model included 3711 measurements from 1412 participants. The predictors were the centre and the interaction of each centre with the standardized seasonal amplitude (SSA). The SSA for this data set is a sinus function reaching its minimum -1.0 on January 20 and its maximum +1.0 on July 21, as explained in the statistical methods section. The participant ID was included as random effect. The fixed effect regression fits are visualized within each plot. The largest seasonal oscillations were

590 observed in Germany (92.1 nmol/l in summer vs. 41.9 nmol/l in winter), and the smallest  
591 in Poland (67.1 nmol/l in summer vs. 50.4 nmol/l in winter). Example calculation: on May  
592 20, the SSA reaches 0.5, therefore the estimate for a participant in Germany would be  
593  $67.0 + 0.5 \times 25.1 = 79.6$  nmol/l. Horizontal lines indicate vitamin D status intervals: < 25 nM  
594 deficient, 25-50 nM insufficient, 50-75 nM sufficient, > 75 nM optimal range.

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598 Figure 3: Comparison between modelled 25-OH-D3 values and actually measured 25-OH-D3  
599 values, based on a leave-one-out cross-validation of the model in Table 2 and Figure 2. Only  
600 participants for whom all three pre-planned DBS-based measurements were available were  
601 included in the validation. For each measured value a prediction was performed by taking  
602 into account the site and the season as fixed factors, and the subject ID as a random factor.  
603 While the two other measurements of the same participant were included each time, the  
604 measurement to be predicted was excluded each time. The resulting Pearson correlation  
605 between measured and modelled values was  $r=0.65$ , and the differences between the  
606 modelled and measured values had a standard deviation of 21.2 nmol/l.

607